

AMENDMENT

Please cancel claims 110- 413 . The pending claims are 1-109 as follows:

1. A non-naturally occurring single chain protein comprising: i) a first polypeptide having a binding domain polypeptide capable of binding to a target molecule, said binding domain polypeptide comprising a heavy chain variable region, said heavy chain variable region comprising an amino acid substitution or deletion at one or more amino acid residues; ii) a second polypeptide comprising a connecting region attached to said first polypeptide; and iii) a third polypeptide comprising an N-terminally truncated immunoglobulin heavy chain constant region polypeptide attached to the second polypeptide, wherein said non-naturally occurring single-chain protein is capable of at least one immunological activity.
2. A protein of claim 1 wherein said binding domain polypeptide is a single chain Fv.
3. A protein of claim 1 wherein the one or more amino acid substitution or deletion in said heavy chain variable region is effective to increase expression or stability of said protein relative to a protein without said deletion or substitution.
4. A protein of claim 1 wherein said binding domain polypeptide comprises an immunoglobulin light chain variable region polypeptide and an immunoglobulin heavy chain variable region polypeptide.
5. A protein of claim 4 further comprising a second binding domain polypeptide capable of binding a second target molecule, said second binding domain polypeptide comprising an immunoglobulin light chain variable region polypeptide and an immunoglobulin heavy chain variable region polypeptide.
6. A protein of claim 5 wherein the first target molecule and the second target molecule are different.
7. A protein of claim 5 wherein the first target molecule and the second target molecule are the same.

8. A protein of claim 1 wherein said binding domain polypeptide is a single chain Fv comprising one or more amino acid substitution in positions 9, 10, 11, 12, 108, 110, 112 in said heavy chain variable region.

9. A protein of claim 1 wherein said binding domain polypeptide is a single chain Fv comprising an amino acid substitution at position 11 in said heavy chain variable region.

10. A protein of claim 9 wherein the amino acid substituted for the amino acid at position of 11 of the single chain Fv heavy chain variable region is selected from the group consisting of serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartic acid, glutamic acid, lysine, arginine, and histidine.

11. A protein of claim 9 wherein the amino acid substituted for the amino acid at position of 11 of the single chain Fv heavy chain variable region is selected from the group consisting of serine, threonine, cysteine, tyrosine, asparagine, and glutamine.

12. A protein of claim 9 where leucine is replaced by serine at position 11.

13. A protein of claim 9 where leucine is replaced by des-leucine at position 11.

14. A protein of claim 12 having an increased recombinant expression or stability relative to said protein not having an amino acid substitution at position 11.

15. A protein of claim 14 wherein the expression of said protein having an amino acid substitution at position 11 is 10-100 fold greater than said protein without a substitution at position 11.

16. A protein of claim 14 wherein said expression is in mammalian cells.

17. A protein of claim 1 wherein said binding domain polypeptide is a single chain Fv and the amino acid at position 11 of the heavy chain variable region of said single chain Fv has been deleted.

18. A protein of claim 1 wherein said binding domain polypeptide is a single chain Fv and said binding domain polypeptide comprises a light chain variable region, wherein said light chain variable region has an amino acid deletion or substitution at one or more of amino acid positions 12, 80, 81, 83, 105, 106, and 107.

19. A protein of claim 18 wherein the amino acid at position 106 has been substituted or deleted.

20. A protein of claim 2 wherein said binding domain polypeptide binds to a tumor antigen.
21. A protein of claim 2 wherein said binding domain polypeptide binds to an antigen on an immune effector cell.
22. A protein of claim 2 wherein said binding domain polypeptide binds to a cancer cell antigen.
23. A protein of claim 22 wherein said cancer cell antigen is a surface antigen.
24. A protein of claim 22 wherein said cancer cell antigen is an intracellular antigen.
25. A protein of claim 1 wherein said binding domain polypeptide binds to a B cell antigen.
26. A protein of claim 25 wherein said B cell antigen is selected from the group consisting of CD19, CD20, CD22, CD37, CD40, CD80, and CD86.
27. A protein of claim 2 wherein said single chain Fv binds to a B cell antigen.
28. A protein of claim 27 wherein said B cell antigen is selected from the group consisting of CD19, CD20, CD22, CD37, CD40, CD80, and CD86.
29. A protein of claim 28 wherein said single chain Fv is selected from the group consisting of HD37 single chain Fv, 2H7 single chain Fv, G28-1 single chain Fv, and 4.4.220 single chain Fv.
30. A protein of claim 2 wherein said single chain Fv is selected from the group consisting of HD37 single chain Fv, 2H7 single chain Fv, G28-1 single chain Fv, FC2-2, UCHL-1, 5B9, L6, 10A8, 2e12, 40.2.36, G19-4, 1D8, and 4.4.220 single chain Fv.
31. A protein of claim 1 wherein said binding domain polypeptide is an scFv that binds to a B cell differentiation antigen.
32. A protein of claim 31 wherein said B cell antigen is selected from the group consisting of CD19, CD20, CD22, CD37, and CD40.
33. A protein of claim 1 wherein said binding domain polypeptide binds to a target selected from the group consisting of CD2, CD3, CD4, CD5, CD6, CD8, CD10, CD11b, CD14, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD28, CD30, CD37, CD40, CD43, CD50 (ICAM3), CD54 (ICAM1), CD56, CD69, CD80, CD86, CD134 (OX40), CD137 (41BB),

CD152 (CTLA-4), CD153 (CD30 ligand), CD154 (CD40 ligand), ICOS, L6, B7-H1, and HLA class II.

34. A protein of claim 1 wherein said protein is capable of forming a complex comprising two or more of said proteins.

35. A protein of claim 34 wherein said complex is a dimer.

36. A protein of claim 1 wherein said protein is a monomer.

37. A protein of claim 1 coupled to a drug, toxin, immunomodulator, polypeptide effector, isotope, label, or effector moiety.

38. A protein of claim 1 wherein said immunological activity is selected from the group consisting of antibody dependent cell-mediated cytotoxicity, complement fixation, induction of apoptosis, induction of one or more biologically active signals, induction of one or more immune effector cells, activation of cellular differentiation, cellular activation, release of one or more biologically active molecules, and neutralization of an infectious agent or toxin.

39. A protein of claim 38 which is capable of induction of biologically active signals by activation or inhibition of one or more molecules selected from the group consisting of protein kinases, protein phosphatases, G-proteins, cyclic nucleotides or other second messengers, ion channels, and secretory pathway components.

40. A protein of claim 38 which is capable of induction of one or more immune effector cells selected from the group consisting of NK cells, monocytes, macrophages, B cells, T cells, mast cells, neutrophils, eosinophils, and basophils.

41. A protein of claim 40 wherein said induction of one or more immune effector cells leads to antibody dependent cell-mediated cytotoxicity or the release of one or more biologically active molecules.

42. A protein of claim 38 which is capable of cellular activation, wherein said activation leads to changes in cellular transcriptional activity.

43. A protein of claim 42 wherein said cellular transcriptional activity is increased.

44. A protein of claim 42 wherein said cellular transcriptional activity is decreased.

45. A protein of claim 38 wherein said one or more biologically active molecules is a protease.

46. A protein of claim 38 wherein said one or more biologically active molecules is a cytokine.

47. A protein of claim 46 wherein said cytokine is selected from the group consisting of monokines, lymphokines, chemokines, growth factors, colony stimulating factors, interferons, and interleukins.

48. A protein of claim 38 which is capable of neutralization of an infectious agent, wherein said infectious agent is a bacterium, a virus, a parasite, or a fungus.

49. A protein of claim 38 which is capable of neutralization of a toxin, wherein said toxin is selected from the group consisting of endotoxins and exotoxins.

50. A protein of claim 38 which is capable of neutralization of a toxin, wherein said toxin is an exotoxin selected from the group consisting of anthrax toxin, cholera toxin, diphtheria toxin, pertussis toxin, *E. coli* heat-labile toxin LT, *E. coli* heat stable toxin ST, shiga toxin *Pseudomonas* Exotoxin A, botulinum toxin, tetanus toxin, *Bordetella pertussis* AC toxin, and *Bacillus anthracis* EF.

51. A protein of claim 38 which is capable of neutralization of a toxin, wherein said toxin is an endotoxin selected from the group consisting of saxitoxins, tetrodotoxin, mushroom toxins, aflatoxins, pyrrolizidine alkaloids, phytohemagglutinins, and grayanotoxins.

52. A protein of claim 1 wherein said protein is capable of binding to an intracellular target to effect a cellular function.

53. A protein of claim 1 wherein said binding domain polypeptide comprises a light chain variable region attached to said heavy chain variable region by a binding domain linker, wherein said binding domain linker comprises one or more peptide having a sequence Gly-Gly-Gly-Gly-Ser.

54. A protein of claim 53 comprising three Gly-Gly-Gly-Gly-Ser peptides.

55. A protein of claim 1 wherein said binding domain polypeptide comprises wild type or engineered immunoglobulin variable region obtained from species selected from the group consisting of human, murine, rat, pig, and monkey.

56. A protein of claim 1 wherein said binding domain polypeptide comprises a humanized immunoglobulin variable region.

57. A protein of claim 2 wherein said N-terminally truncated immunoglobulin heavy chain constant region polypeptide comprises an IgG CH2 constant region polypeptide attached to an immunoglobulin heavy chain IgG CH3 constant region polypeptide.

58. A protein of claim 2 wherein said N-terminally truncated immunoglobulin heavy chain constant region polypeptide consist essentially of an IgG CH2 constant region polypeptide attached to an immunoglobulin heavy chain IgG CH3 constant region polypeptide.

59. A protein of claim 2 wherein said N-terminally truncated immunoglobulin heavy chain constant region polypeptide comprises an IgG CH2 constant region polypeptide attached to an immunoglobulin heavy chain IgG CH3 constant region polypeptide.

60. A protein of claim 2 wherein said N-terminally truncated immunoglobulin heavy chain constant region polypeptide consist essentially of an IgG CH2 constant region polypeptide attached to an immunoglobulin heavy chain IgG CH3 constant region polypeptide.

61. A protein of claim 1 wherein said binding domain polypeptide is a single chain Fv that comprises at least a portion of a human constant region.

62. A protein of claim 2 wherein said binding domain polypeptide is a single chain Fv that comprises at least a portion of a human constant region.

63. A protein of claim 1 wherein said connecting region comprises a naturally occurring hinge region selected from the group consisting of a human hinge or portion thereof, human IgG hinge or a portion thereof, human IgA hinge or a portion thereof, human IgE hinge or a portion thereof, camelid hinge region or a portion thereof, IgG1 llama hinge region or portion thereof, nurse shark hinge region or portion thereof, and spotted ratfish hinge region or a portion thereof.

64. A protein of claim 1 wherein said connecting region comprises a human IgE hinge or a portion thereof.

65. A protein of claim 1 wherein said connecting region comprises a human IgG1, IgG2, IgG3 or IgG4 hinge region having either zero or one cysteine residue.

66. A protein of claim 1 wherein said connecting region comprises a human IgGA hinge region having between zero and two cysteine residues.

67. A protein of claim 1 wherein said connecting region comprises a wild type human IgG1 immunoglobulin hinge region.

68. A protein of claim 1 wherein said connecting region comprises a glycosylation site.

69. A protein of claim 1 wherein said connecting region has no cysteine residues capable of forming disulfide bonds.

70. A protein of claim 1 wherein said connecting region has one cysteine residue.

71. A protein of claim 1 wherein said connecting region comprises a mutated wild-type immunoglobulin hinge region polypeptide comprising no more than one cysteine residue.

72. A protein of claim 1 wherein said connecting region is altered so that said protein has a reduced ability to dimerize.

73. A protein of claim 1 where said connecting region comprises three cysteine residues and one proline residue, wherein one or more of said cysteine residues is deleted or substituted and said proline residue is substituted or deleted.

74. A protein of claim 1 wherein said connecting region comprises a mutated wild-type immunoglobulin hinge region polypeptide comprising first, second, and third cysteine residues, where said first cysteine residue is N-terminal to said second cysteine and said second cysteine is N-terminal to said third cysteine, wherein said first cysteine residue is substituted or deleted.

75. A protein of claim 74 wherein said wild-type hinge region polypeptide is from human IgG1.

76. A non-naturally occurring single chain protein comprising: i) a first polypeptide having a binding domain polypeptide capable of binding to a target molecule, said binding domain polypeptide comprising a heavy chain variable region comprising one or more amino acid deletion or substitution in positions 9, 10, 11, 12, 108, 110, 112; ii) a second polypeptide comprising a connecting region attached to said first polypeptide; and iii) a third polypeptide

comprising an N-terminally truncated immunoglobulin heavy chain constant region polypeptide attached to the second polypeptide, wherein said non-naturally occurring single-chain protein is capable of at least one immunological activity.

77. A non-naturally occurring single chain Fv protein comprising: i) a first polypeptide having a binding domain polypeptide capable of binding to a target molecule, said binding domain polypeptide comprising a heavy chain variable region wherein leucine is replaced by serine at position 11 in the first framework region of the heavy chain variable region; ii) a second polypeptide comprising a connecting region attached to said first polypeptide; and iii) a third polypeptide comprising an N-terminally truncated immunoglobulin heavy chain constant region polypeptide attached to the second polypeptide, wherein said non-naturally occurring single-chain protein is capable of at least one immunological activity.

78. A protein of claim 77 further comprising a substitution or deletion of the amino acid at position 10 in the first framework region of the heavy chain variable region.

79. A protein of claim 77 wherein the amino acid substitution at position 11 is effective to increase expression or stability of said single chain Fv protein relative to a single chain Fv protein without said deletion or substitution.

80. A protein of claim 77 wherein the numbering of the residues is the EU index according to Kabat.

81. A protein of claim 77 wherein said connecting region comprises a proline and first, second, and third cysteine residues, where said first cysteine residue is N-terminal to said second cysteine and said second cysteine is N-terminal to said third cysteine, said third cysteine residue is N-terminal to said proline residue.

82. A protein of claim 77 wherein said connecting region comprises an IgA hinge region or portion thereof.

83. A protein of claim 81 where said single chain protein comprises a single chain Fv binding domain from a 2H7 hybridoma, wherein said second cysteine residue is replaced by

serine and said proline residue is replaced by serine in the connecting region, and said heavy chain constant region comprises CH2 and CH3 domains from IgG₁.

84. A protein of claim 82 wherein said single chain protein comprises a single chain Fv binding domain from a 2H7 hybridoma, said connecting region comprises a murine IgA hinge or portion thereof, and said heavy chain constant region comprises CH2 and CH3 domains from murine IgA, wherein said CH3 comprises a deletion or substitution in four amino acids that render the IgA heavy chain constant region incapable of associating with a J chain polypeptide.

85. A protein of claim 81 where said single chain protein comprises a 2H7 single chain Fv binding domain, wherein said first, second, and third cysteine residues in the connecting region are replaced by serine and said proline residue in the connecting region is replaced by serine, said heavy chain constant region comprising CH2 and CH3 domains from IgG₁ wherein lysine is replaced by serine at position 322 in said CH2 region.

86. A protein of claim 81 where said single chain protein comprises a single chain Fv binding domain from a 2H7 hybridoma, wherein in the connecting region said second and third cysteine residues are replaced by serine and said proline residue is replaced by serine, said heavy chain constant region comprising CH2 and CH3 domains from IgG₁ wherein lysine is replaced by serine at position 322 in said CH2 region.

87. A protein of claim 81 where said single chain protein comprises a single chain Fv binding domain from a 2H7 hybridoma, wherein in the connecting region said first, second, and third cysteine residues are replaced by serine and said proline residue is replaced by serine, said heavy chain constant region comprising CH2 and CH3 domains from IgG₁ wherein proline is replaced by serine at position 331 in said CH2 region.

88. A protein of claim 81 where said single chain protein comprises a single chain Fv binding domain from a 2H7 hybridoma, wherein in the connecting region said second and third cysteine residues are replaced by serine and said proline residue is replaced by serine, said heavy chain constant region comprising CH2 and CH3 domains from IgG₁ wherein proline is replaced by serine at position 331 in said CH2 region.

89. A protein of claim 81 where said single chain protein comprises a 2H7 single chain Fv binding domain, wherein in the connecting region said first, second, and third cysteine residues are replaced by serine and said proline residue is replaced by serine, said heavy chain constant region comprising CH2 and CH3 domains from IgG₁ wherein threonine is replaced by asparagine at position 256 in said CH2 region.

90. A protein of claim 81 where said single chain protein comprises a 2H7 single chain Fv binding domain, where in the connecting region said first, second, and third cysteine residues are replaced by serine and said proline residue is replaced by serine, said heavy chain constant region comprises 2H7 single chain Fv CH2 and CH3 domains from IgG₁ where in the CH2 domain arginine is replaced by glutamine at position 255, threonine is replaced by asparagine at position 256, proline is replaced by alanine at position 257, and glutamic acid is replaced by lysine at position 258.

91. A protein of claim 81 where said single chain protein comprises a single chain Fv binding domain from a 2H7 hybridoma, where in the connecting region said first, second and third cysteine residues are replaced by serine and said proline residue is replaced by serine, said heavy chain constant region comprising CH2 and CH3 domains from IgG₁ wherein lysine is replaced by glutamine at position 290 in said CH2 region.

92. A protein of claim 81 where said single chain protein comprises a single chain Fv binding domain from a 2H7 hybridoma, where in the connecting region said first, second and third cysteine residues are replaced by serine and said proline residue is replaced by serine, said heavy chain constant region comprising CH2 and CH3 domains from IgG₁ wherein alanine is replaced by proline at position 339 in said CH2 region.

93. A protein of claim 81 where said single chain protein comprises a single chain Fv binding domain from a G28-1 hybridoma, where in the connecting region said first, second, and third cysteine residues are replaced by serine and said proline residue is replaced by serine, said heavy chain constant region comprising CH2 and CH3 domains from IgG₁.

94. A protein of claim 81 where said single chain protein comprises a single chain Fv binding domain from a G28-1 hybridoma, where in the connecting region said second and third

cysteine residues are replaced by serine and said proline residue is replaced by serine, said heavy chain constant region comprising CH2 and CH3 domains from IgG₁.

95. A protein of claim 81 where said single chain protein comprises a single chain Fv binding domain from a G28-1 hybridoma, where in the connecting region said second cysteine residue is replaced by serine and said proline residue is replaced by serine, said heavy chain constant region comprising CH2 and CH3 domains from IgG₁.

96. A protein of claim 81 said single chain protein comprises a single chain Fv binding domain from a G28-1 hybridoma, where in the connecting region said first and second cysteine residues are replaced by serine, said heavy chain constant region comprising CH2 and CH3 domains from IgG₁.

97. A protein of claim 81 said single chain protein comprises a single chain Fv binding domain from a FC2-2 hybridoma, where in the connecting region said first, second, and third cysteine residues are replaced by serine and said proline residue is replaced by serine, said heavy chain constant region comprising CH2 and CH3 domains from IgG₁.

98. A protein of claim 81 where said single chain protein comprises a single chain Fv binding domain from a UCHL-1 hybridoma, where in the connecting region said first, second, and third cysteine residues are replaced by serine and said proline residue is replaced by serine, said heavy chain constant region comprising CH2 and CH3 domains from IgG₁.

99. A protein of claim 81 where said single chain protein comprises a single chain Fv binding domain from a 5B9 hybridoma, where in the connecting region said first, second, and third cysteine residues are replaced by serine and said proline residue is replaced by serine, where said heavy chain constant region comprises CH2 and CH3 domains from IgG₁.

100. A protein of claim 81 where said single chain protein comprises a single chain Fv binding domain from a 2H7 hybridoma, where in the connecting region said first, second and third cysteine residues are replaced by serine and said proline residue is replaced by serine, said heavy chain constant region comprising CH2 and CH3 domains from IgG₁.

101. A protein of claim 81 where said single chain protein comprises a single chain Fv binding domain from a 2H7 hybridoma, where in the connecting region said second and third cysteine residues are replaced by serine and said proline residue is replaced by serine, where said heavy chain constant region comprises CH2 and CH3 domains from IgG₁.

102. A protein of claim 81 where said single chain protein comprises a single chain Fv binding domain from a G28-1 hybridoma, where in the connecting region said first and third cysteine residues are replaced by serine and said proline residue is replaced by serine, said heavy chain constant region comprising CH2 and CH3 domains from IgG₁.

103. A protein of claim 81 where said single chain protein comprises a single chain Fv binding domain from a G28-1 hybridoma comprising a connecting region wherein said third cysteine residue is replaced by serine and said proline residue is replaced by serine, said heavy chain constant region comprising CH2 and CH3 domains from IgG₁.

104. A protein of claim 81 where said single chain protein comprises a single chain Fv binding domain from a G28-1 hybridoma, where in the connecting region said first cysteine residue is replaced by serine, said heavy chain constant region comprising CH2 and CH3 domains from IgG₁.

105. A protein of claim 82 wherein said single chain protein comprises a single chain Fv binding domain from a G28-1 hybridoma, wherein said connecting region comprising a murine IgA hinge region, said heavy chain constant region comprises CH2 and CH3 domains from murine IgA and said CH3 comprises a deletion or substitution in four amino acids that render the IgA heavy chain constant region incapable of associating with a J chain polypeptide.

106. A protein of claim 82 wherein said single chain protein comprises a single chain Fv binding domain from a G28-1 hybridoma, wherein said connecting region comprising a human IgA hinge region and said heavy chain constant region comprises CH2 and CH3 domains from human IgA, where said CH3 comprises a deletion or substitution in four amino acids that render the IgA heavy chain constant region incapable of associating with a J chain polypeptide.

107. A protein of claim 81 where said single chain protein comprises a single chain Fv binding domain from a HD37 hybridoma, where in the connecting region said first, second, and

third cysteine residues are replaced by serine and said proline residue is replaced by serine, and said heavy chain constant region comprising CH2 and CH3 domains from IgG₁.

108. A protein of claim 81 where said single chain protein comprises a L6 single chain Fv binding domain, where in the connecting region said first, second, and third cysteine residues are replaced by serine and said proline residue is replaced by serine, and said heavy chain constant region comprises CH2 and CH3 domains from IgG₁.

109. A non-naturally occurring single chain Fv protein comprising: i) a first polypeptide having a binding domain polypeptide capable of binding to a target molecule, said binding domain polypeptide comprising a heavy chain variable region where leucine is replaced by serine at position 11 in the first framework region of the heavy chain variable region; wherein said protein has an increased expression or stability in mammalian cells relative to a protein not having said amino acid substitution; ii) a second polypeptide comprising a connecting region attached to said first polypeptide; and iii) a third polypeptide comprising an N-terminally truncated immunoglobulin heavy chain constant region polypeptide attached to the second polypeptide, wherein said non-naturally occurring single-chain Fv protein is capable of at least one immunological activity.

CONCLUSION

Applicants request entry of this Preliminary Amendment. Applicants respectfully submit that all pending claims are in condition for allowance.

Respectfully submitted,

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